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STATE-OF-THE-ART REVIEW

FLIP as a therapeutic target in cancer

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One of the classic hallmarks of cancer is disruption of cell death signalling. Inhibition of cell death promotes tumour growth and metastasis, causes resistance to chemo- and radiotherapies as well as targeted agents, and is frequently due to overexpression of antiapoptotic proteins rather than loss of pro-apoptotic effectors. FLIP is a major apoptosis-regulatory protein frequently overexpressed in solid and haematological cancers, in which its high expression is often correlated with poor prognosis. FLIP, which is expressed as long (FLIP(L)) and short (FLIP(S)) splice forms, achieves its cell death regulatory functions by binding to FADD, a critical adaptor protein which links FLIP to the apical caspase in the extrinsic apoptotic pathway, caspase-8, in a number of cell death regulating complexes, such as the death-inducing signalling complexes (DISCs) formed by death receptors. FLIP also plays a key role (together with caspase-8) in regulating another form of cell death termed programmed necrosis or 'necroptosis', as well as in other key cellular processes that impact cell survival, including autophagy. In addition, FLIP impacts activation of the intrinsic mitochondrial-mediated apoptotic pathway by regulating caspase-8-mediated activation of the pro-apoptotic Bcl-2 family member Bid. It has been demonstrated that FLIP can not only inhibit death receptor-mediated

Abbreviations
AIM2, absent in melanoma-2; AP1, activator protein 1; ASC, apoptosis-associated Speck-like protein containing a CARD; Ask1, apoptosis-signal-regulating-kinase-1; Atg 3, autophagy related 3; Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2-associated X protein; Bcl-2, B-Cell Lymphoma 2; BCLAF, Bcl-2-associated transcription factor; BET, bromodomain and extraterminal; Bid, BH3 interacting-domain death agonist; CAMK, calcium/calmodulin-dependent protein kinase; CARD, caspase recruitment domain; Cas-9, CRISPR-associated protein-9; CASP8, Caspase-8; CDK, cyclin-dependent kinase; CHOP, C/EBP homologous protein; CREB, cAMP response element binding; CRISPR, Clustered Regulatory Interspaced Short Palindromic Repeats; CTL, cytotoxic lymphocyte; DED, death effector domain; DISC, death-inducing signalling complex; DUB, deubiquitinase; E2F1, E2F transcription factor 1; EGR 1, early growth response-1; ER, endoplasmic reticulum; ERK, extracellular-signalling Regulated Kinase; FADD, Fas-associated protein with death domain; FDA, food and drug administration; FLIP, FADD-like IL1-β-converting enzyme-inhibitory protein; GMCSF, granulocyte-macrophage colony-stimulating factor; HDAC, histone deacetylase; HIF, hypoxia inducible factor; HNSCC, head and neck squamous cell carcinoma; IAP, inhibitor of apoptosis protein; IKK, IkappaB kinase complex; IL, interleukin; IRE-1, inositol requiring-1; IRF5, interferon regulatory factor-5; ITF2, immunoglobulin transcription factor-2; JNK, Jun N-terminal kinase; KSHV, Kaposi's sarcoma-associated herpes virus; LC3, microtubule-associated protein 1A/1B-light chain 3; LPS, lipopolysaccharide; MAM, mitochondrial-associated membrane; MAPK, mitogen-activated protein kinase; Mcl-1, myeloid cell leukaemia-1; MDSC, myeloid derived suppressor cell; MEF, mouse embryonic fibroblast; MEKK, MAP/ERK kinase kinase-1; MLKL, mixed lineage kinase domain like pseudo-kinase; MM, multiple myeloma; MOMP, mitochondrial outer membrane permeabilization; mTOR, mammalian target of rapamycin; NEMO, NFκB essential modulator; NES, nuclear export signal; NFAT, nuclear factor of activated T cells; NFκB, nuclear factor kappa-light-chain-enhancer of B-cell; NLRP3, NLR family pyrin domain containing-3; NLS, nuclear localization signal; NO, nitric oxide; NSCLC, non-small-cell lung cancer; PERK, PKR-like ER kinase; PKC, protein kinase C; RalBP1, Ral A binding protein-1; RIP, receptor-interacting kinase; ROS, reactive oxygen species; RTN, reticulon; S6K1, S6 kinase-1; SIRT-1, Sirtuin-1; SMAC, second mitochondria-derived activator of caspases; TCGA, The Cancer Genome Atlas; TEN, toxic epidermal neolysis; TGF, transforming growth factor; TIP49, TBP-interacting protein-49; TNF, tumour necrosis factor; TRADD, TNFR1-associated death domain protein; TRAF, TNF receptor-associated factor; TRAIL, TNF-related apoptosis-inducing ligand; UPS, ubiquitin proteasome system; USP, ubiquitin-specific protease; XIAP, X-linked inhibitor of apoptosis protein.

apoptosis, but also cell death induced by a range of clinically relevant chemotherapeutic and targeted agents as well as ionizing radiation. More recently, key roles for FLIP in promoting the survival of immunosuppressive tumour-promoting immune cells have been discovered. Thus, FLIP is of significant interest as an anticancer therapeutic target. In this article, we review FLIP's biology and potential ways of targeting this important tumour and immune cell death regulator.

Introduction

The major limitation to the success of current cancer therapies is resistance mechanisms that are either present prior to treatment (intrinsic) or develop or are selected for during treatment (acquired). Defects in cell death and survival signalling pathways in malignant cells contribute to drug resistance [1]. Over the past decade, The Cancer Genome Atlas (TCGA) has described genomic alterations in apoptotic proteins, including amplification and overexpression of anti-apoptotic proteins such as the IAPs (inhibitors of apoptosis proteins) and mutations and loss of expression of cell death effectors, including caspase-encoding genes. Therefore, evasion of apoptosis is considered a hallmark of cancer and it is the rationale behind the design of novel and more effective anticancer therapies targeting the cell death machinery [2].

Best known for its role in regulating caspase-8, FLIP (Fas-associated death domain (FADD)-like IL-1β-converting enzyme-inhibitory protein) is a multifunctional protein with roles in regulating important cellular processes such as apoptosis, necroptosis, autophagy and inflammation [3]. FLIP also has roles in innate and adaptive immunity and during embryonic development [4–7]. In cancer, upregulation of FLIP has been reported to contribute to tumour progression in prostate, colorectal, bladder urothelial, cervical, hepatocellular and gastric cancers, Head and Neck Squamous Cell Carcinoma (HNSCC), and in Burkitt's and non-Hodgkin's lymphomas. FLIP overexpression also correlates with a poor clinical outcome in cancers such as colorectal and non-small-cell lung cancer (NSCLC). The association with poor prognosis and disease progression are likely related to FLIP's cell death inhibitory function; therefore, FLIP is potentially a relevant prognostic biomarker and therapeutic target in several cancer cell types [8–10].

Here, we review the various cellular roles of FLIP and its relevance in cancer, and we discuss several approaches to therapeutically target this important cell death regulatory protein.

Canonical FLIP Biology: regulation of caspase-8 activation

FLIP splice forms

The gene encoding FLIP (*CFLAR*) is located on chromosome 2q33.1 in a region adjacent to the genes encoding its paralogs caspase-8 (*CASP8*) and caspase-10 (*CASP10*). FLIP is primarily expressed as long (FLIP(L)) and short (FLIP(S)) splice variants in human cells. Both splice variants have two tandemly arranged protein–protein interaction domains called Death Effector Domains (DEDs) (Fig. 1A). In FLIP(L), the C-terminal region closely resembles the equivalent regions of caspase-8 and caspase-10, but does not contain a functional caspase domain. In FLIP(S), there is no pseudo-caspase domain at the C terminus; instead, there is a unique, short unstructured sequence that is involved in its turnover via the ubiquitin-proteasome system. A third splice form FLIP(R) is expressed in some tissues; similar in size and structure to FLIP(S), it has a different C-terminal region. FLIP(L) contains a caspase-8 cleavage site at position Asp-376, which produces a proteolytic N-terminal fragment p43-FLIP(L) when the two proteins form heterodimers. FLIP proteins are also encoded by a number of viruses, including *molluscum contagiosum* and Kaposi's sarcoma-associated herpesvirus; such proteins share similar structures as their cellular counterparts and modulate cell survival and innate immunity [11–15].

FLIP and the DISC

The extrinsic apoptotic pathway is triggered when the TNF receptor superfamily members TRAIL-R1/DR4, TRAIL-R2/DR5 and Fas/CD95 are bound by their cognate ligands (TRAIL and FasL, respectively; Fig. 2A). This leads to recruitment of the adapter protein FADD and formation of the death-inducing signalling complex (DISC). Into this complex, FADD recruits procaspase-8 via homotypic DED-mediated

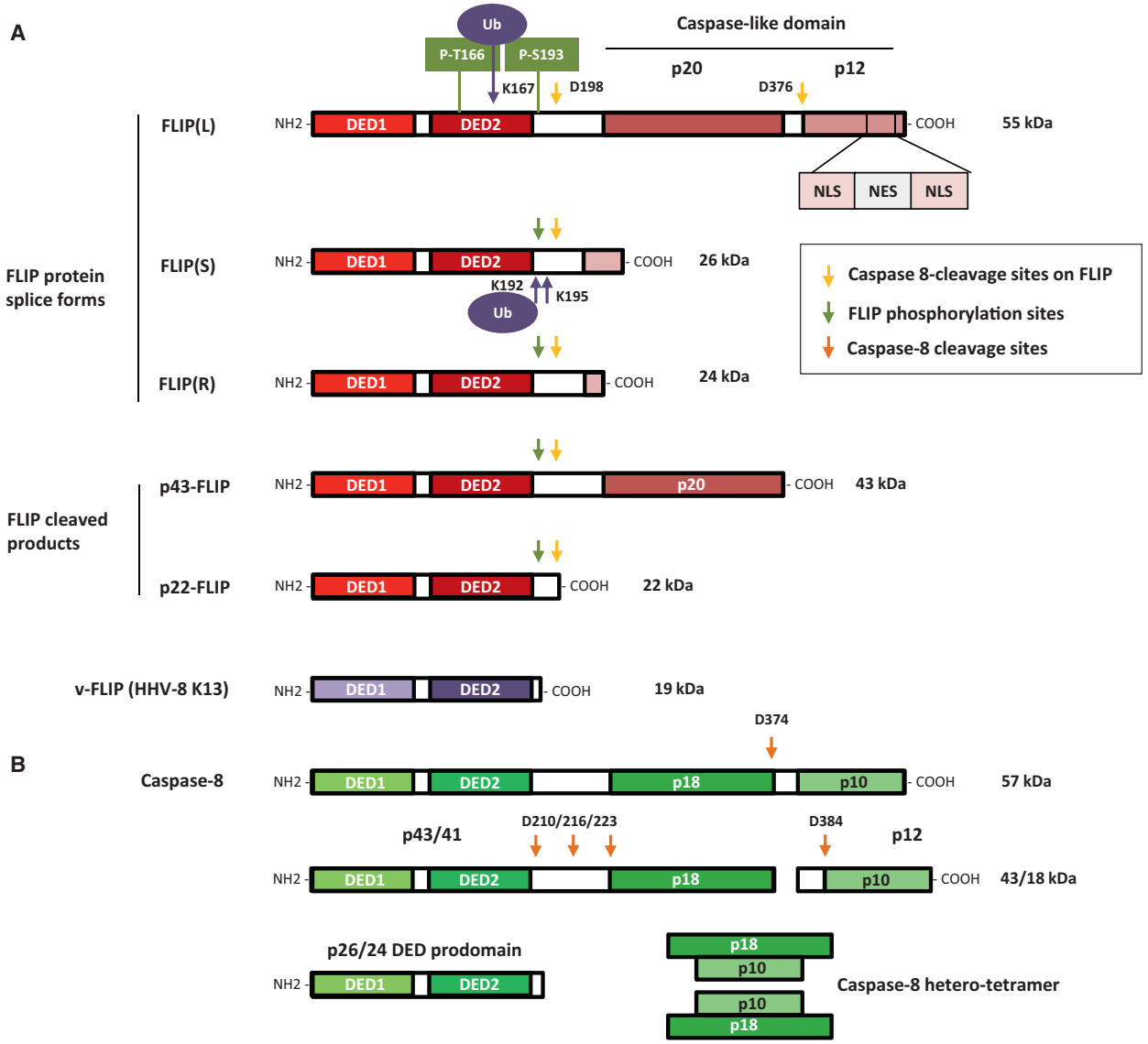

Fig. 1. (A) Schematic representation of cellular and viral FLIP splice variants. All splice variants contain tandem DEDs allowing recruitment to the DISC. FLIP(L) has a pseudo-kinase domain that can be cleaved by DISC-bound procaspase-8 at Asp 376 to form p43- and p12-cleavage products. The FLIP(L) pseudo-kinase domain also contains a nuclear localization signal (NLS) and nuclear export signal (NES). FLIP (S/R) lack the pseudo-caspase domain of FLIP(L) and differ from each other in their C terminus. Viral homologues of FLIP have also been identified which have tandem DED domains and are similar in structure to FLIP(S)/(R). (B) Procaspase-8 structure and processing. Dimerization of procaspase-8 leads to an initial cleavage in the catalytic domain at Asp374, producing a p12 and p43/41 cleavage product. Further processing at the indicated aspartic acid residues generates the p10- and p18-cleavage products which make up the apoptosis-active caspase-8 hetero-tetramer. In addition, the p26/p24 DED pro-domain is also generated during this processing.

protein:protein interactions, leading to formation of procaspase-8 homodimers, which is an essential step in its enzymatic activation [16,17]. Initial cleavage of procaspase-8 occurs at Asp 374 in an inter-dimer manner producing p43/41 and p12 subunits (Figs 1B and 2A). Subsequently, intra-dimer cleavage occurs after Asp-210, 216 or 223 and Asp-384 generating the two p18- and two p10-active subunits of the enzyme [18,19]. While the hetero-tetramer of caspase-8 is released from the membrane-bound DISC, the pro-domains of caspase-8 can remain bound: these catalytically inactive DEDs are thought to act as a negative feedback loop

to limit further procaspase-8 recruitment and caspase activation [20].

FADD-like IL1-β-converting enzyme-inhibitory protein can also be recruited to the DISC, where it heterodimerizes with procaspase-8; this alters the processing and activation of procaspase-8 depending on which splice form is recruited. FLIP(S) and FLIP(R) act as simple inhibitors of procaspase-8 processing by inhibiting formation of procaspase-8 homodimers. The role of FLIP(L) is more complex; heterodimerization with procaspase-8 results in the formation of an active enzymatic complex that can promote cleavage of both adjacent procaspase-8 homodimers (and is therefore potentially activatory) and adjacent FLIP(L)/procaspase-8 heterodimers, but which itself has no direct apoptosis-inducing activity (i.e. cannot cleave pro-apoptotic effectors such as Bid and procaspase-3) [21–23] (Fig. 3). If high levels of FLIP(L) are recruited to the DISC, this can be apoptosis inhibitory as the numbers of procaspase-8 homodimers that can form under these conditions will be proportionally lower; however, the stoichiometry at which FLIP(L) changes from being activatory to inhibitory at the DISC has not yet been established [24]. Indeed, DISC stoichiometry has been a matter of intense research in recent years, with several studies reporting that FADD and FLIP are substoichiometric with respect to procaspase-8 [16,17], although the exact stoichiometry remains a matter of debate. All the current models propose that chains containing procaspase-8 and FLIP are formed following DISC stimulation; it is the length of these chains and whether they require just a single 'nucleating' FADD DED or bridging interactions between FADD DEDs bound to adjacent receptors that remain unresolved. An interesting further development is the proposal that parallel DED chains can intertwine to generate 'DED filaments' [25]. Downstream of caspase-8 activation, apoptosis can be induced directly by activation of executioner caspases-3/7 (in so-called Type-I cells) or more usually by amplification via the intrinsic mitochondrial-mediated apoptotic pathway (in Type-II cells) by cleavage of the Bcl-2 family member Bid which can promote mitochondrial outer membrane permeabilization (MOMP) by interacting with other members of the Bcl-2 family, Bax and Bak (Fig. 2). The ratio of caspase-3 and X-linked inhibitor of apoptosis protein (XIAP) is also important for determining the Type-I or Type-II phenotype. XIAP can directly bind to and inhibit caspase-3. High ratios of XIAP:Caspase-3 promote a Type-II phenotype as release of the endogenous XIAP inhibitor second mitochondria-derived activator of caspases (SMAC) from the mitochondria is required to allow caspase-3 activation [26–29]. It is important to note, therefore, that the high levels of XIAP or antiapoptotic Bcl-2 family proteins (such as Bcl-XL, Mcl-1 and Bcl-2 itself) may inhibit apoptosis induction downstream of caspase-8 activation.

Procaspase-10 is another tandem DED-containing caspase that has 4 different splice forms (10A, 10B, 10D, 10G) [30]. It shares significant homology (46% identical) with procaspase-8 in the catalytic domain [31], which has led to the assumption that caspase-8 and caspase-10 serve redundant roles in death receptor-mediated apoptosis. Early experiments demonstrated that procaspase-10 is recruited to the Fas and TRAIL DISCs; however, it was not clear whether it could functionally substitute for caspase-8 in these complexes [32,33]. Overexpression of caspase 10A and 10D re-sensitized caspase-8 knockdown cells to TRAIL-induced apoptosis, whereas 10B and 10G had no effect or were weakly apoptotic, suggesting that splice forms 10A and 10D are functionally important in DISC-mediated apoptosis [30]. More recently, a hierarchical and cooperative model of procaspase-10 recruitment to the DISC has been proposed, requiring initial recruitment of procaspase-8 before procaspase-10 (and indeed FLIP) can bind at the DISC [24]. This has been confirmed in our lab using several CRISPR/Cas9 caspase-8 knockout lines (D. B. Longley, unpublished observations). Interestingly, caspase-10 has been recently shown to inhibit caspase-8 activation, supporting earlier research that reported that the caspase-8/-10 heterodimer is inactive [34,35]. Therefore, in overexpression models, caspase-10 can induce cell death, whereas at physiologically relevant expression levels, it seems to inhibit caspase-8-mediated apoptosis. More research into the function of caspase-10 at the DISC (potentially using splice form-specific siRNAs) is required to understand the exact conditions when caspase-10 can exert these different signalling outcomes. In concert with FLIP(L), procaspase-10 can also regulate autophagy (*see below*).

TNF receptor complex II and the ripoptosome

The tumour necrosis factor receptor-1 (TNFR1) is another member of the TNF receptor superfamily. The downstream signalling events of TNFR1 activation differ from that of TRAIL receptors and Fas (Fig. 2B). TNFR1 recruits a different adapter protein, TNFR1-associated death domain protein (TRADD), which recruits an initial complex termed Complex-I [36] that primarily leads to activation of NFκB and MAP kinase signalling pathways; this has been extensively reviewed elsewhere [37]. Complex-IIa is formed

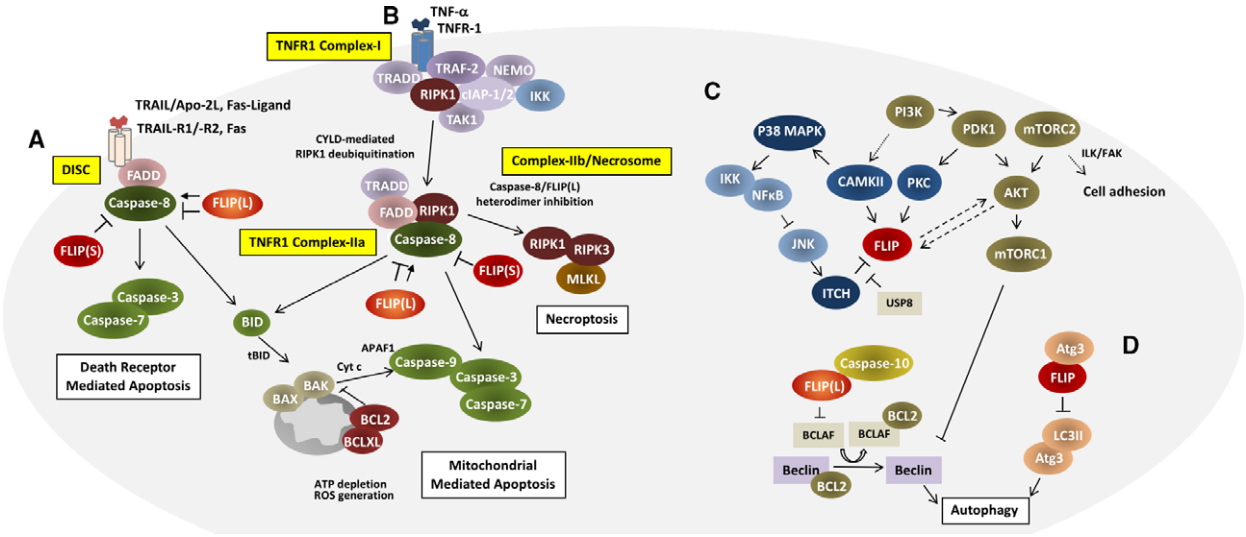

Fig. 2. Schematic overview of molecular mechanisms modulated by FLIP. (A) Death receptor-mediated apoptosis. Activation of cell death receptors (TRAIL-R1/R2, or Fas) initiates formation of the DISC and activation of the extrinsic signalling pathways through FADD-dependent activation of caspase-8; this is regulated by FLIP. (B) TNFα-induced apoptotic and necroptotic cell death. Upon ligation, TNFR1 recruits TRADD, RIPK1, TRAF2 and cIAP1/2 to form TNFR1 Complex-I, which activates NFκB. The deubiquitination of RIPK1 by CYLD leads to formation of cytosolic Complex-II, similar in composition to the DISC into which procaspase-8 can be recruited and activated; like the DISC, this is regulated by FLIP in a splice form-specific manner. The caspase-8/FLIP(L) heterodimer cannot cleave procaspases-3/7 or BID, but can cleave RIPK1; however, if this activity is repressed, RIPK1 forms the necrosome with RIPK3, which in turn activates MLKL leading to necroptosis. (C) Regulatory mechanisms of FLIP. Several protein kinases have been reported to upregulate and/or phosphorylate FLIP, among them CAMKII, PKC, PI3K and AKT. Moreover, in response to various cellular stresses, activation of JNK signalling can promote proteasome-mediated FLIP degradation by the E3 ubiquitin ligase ITCH. Alternatively, USP8, a specific FLIP(L) deubiquitinase, stabilizes FLIP (L) by preventing UPS mediated degradation. (D) Autophagy. FLIP suppresses autophagosome formation by two different mechanisms: (i) inhibiting the interaction between Bcl-2 and Beclin as part of an enzymatic complex with caspase-10 that cleaves the autophagy-inducing BCLAF1; and (ii) by direct interaction with Atg3 thereby inhibiting autophagosome formation.

when TRADD and another death domain-containing protein receptor-interacting kinase (RIPK1) dissociate from TNFR1 Complex-I and recruit FADD, FLIP and procaspase-8 through homotypic DD and DED interactions in a similar way to DISC formation [38]. As an NFκB-target gene (see below), FLIP is upregulated downstream of TNFR1 Complex-I; this upregulation is an important determinant of the signalling outputs from Complex-II. As well as being able to process adjacent homo- (caspase-8/caspase-8) and hetero- (caspase-8/FLIP(L)) dimers, the caspase-8/FLIP (L) heterodimer can also cleave RIPK1, resulting in disassembly of complex IIa thereby preventing formation of Complex-IIb (also referred to as the necrosome) [39]. In Complex-IIb, RIPK1 interacts with RIPK3 to activate mixed lineage kinase domain like pseudo-kinase (MLKL), leading to its translocation to the plasma membrane, where it oligomerizes and forms pores in the membrane eliciting a pro-inflammatory, necrotic form of cell death termed necroptosis [40–42].

The ripoptosome is a 2MDa complex that forms as a result of genotoxic stress or loss of cIAPs 1 and 2 following treatment with IAP antagonists [43,44]. The complex, consisting of RIPK1, FADD, procaspase-8 and FLIP, is distinct from TNFR1-dependent Complex-II as TNFR1 is not required for its formation [45]. However, just as in TNFR1 Complex-II, FLIP has an important role in determining the signalling outcome of the ripoptosome by regulating the proportion of procaspase-8 homodimers relative to FLIP/procaspase-8 heterodimers, which in turn impacts on whether sufficient caspase-8 activity is generated to drive apoptotic cell death and whether RIPK1 is cleaved to inhibit necroptosis [45] (Fig. 3).

In vivo models of FLIP

In contrast to humans, mice only have 2 splice variants of FLIP: FLIP(L) and FLIP(R) [46]. Genetic deletion of FLIP causes mouse embryos to terminate at day 10.5 [47], a phenotype which is reminiscent of those observed upon deletion of the genes encoding caspase-8 (*Casp8*) and FADD (*Fadd*) [48,49].In addition, while deletion of *Casp8* led to embryonic lethality

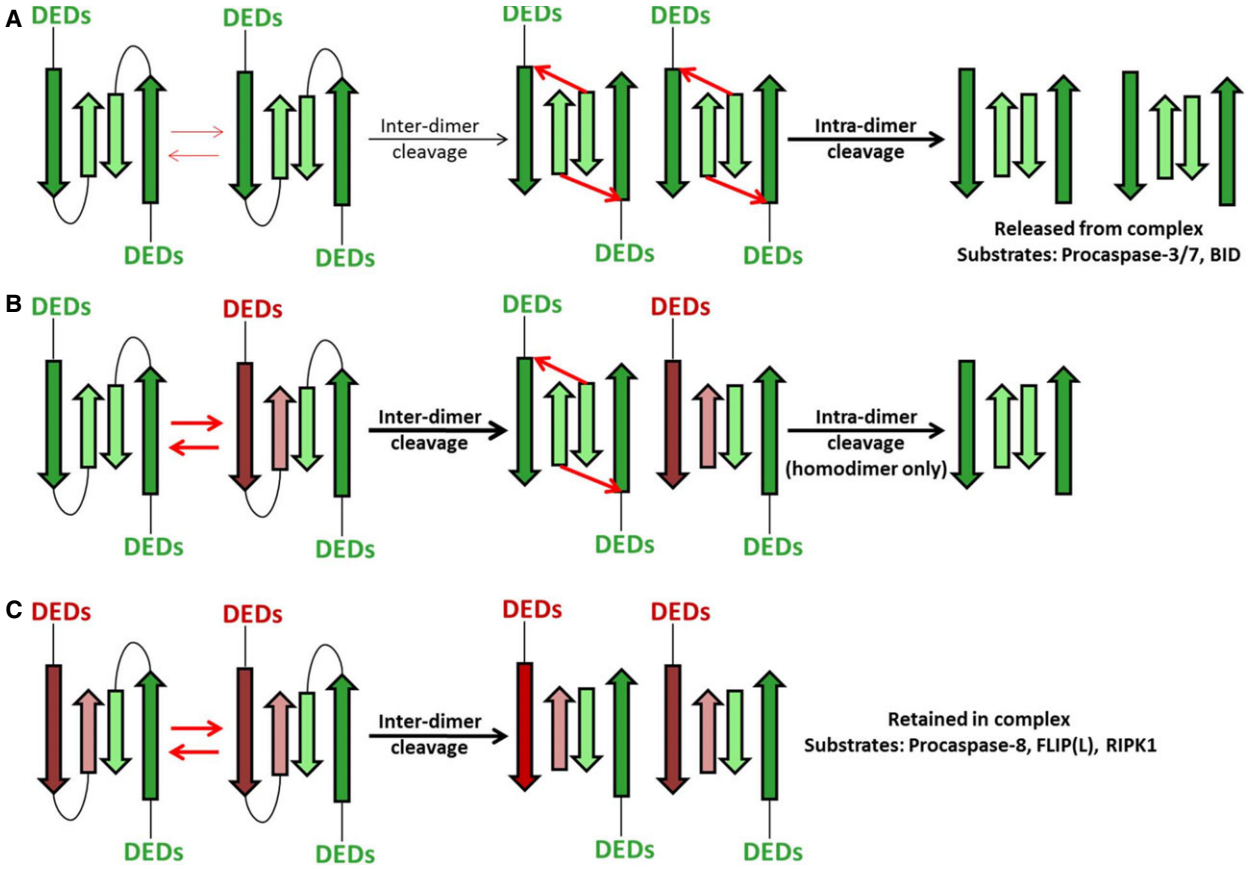

Fig. 3. Schematic overview of procaspase-8 activation at the DISC, TNFR1 Complex II and the ripoptosome. (A) Within a homodimer, the caspase-8 catalytic domains are arranged in an antiparallel fashion and primarily interact via 4 hydrogen bonds between their small p10 catalytic subunits. This creates an enzymatic active site, which can then cleave adjacent homodimers in the region between the large (p18) and small (p10) catalytic subunits. This cleavage enables the 2nd activation step, which is intra-dimer cleavage in the linker region between p18 and the DEDs. The p18/p10 hetero-tetramers that are formed can be released from the complex and activate downstream apoptosis-inducing substrates, procaspases-3/7 and BID. (B) Formation of a heterodimer with FLIP(L) is energetically favourable compared with caspase-8 homodimers as 8 hydrogen bonds can form between the small subunits. This promotes formation of a FLIP(L)/caspase-8 heterodimeric enzyme that can efficiently cleave adjacent caspase-8 homodimers, thereby promoting processing of these homodimers. The lack of critical cysteine in FLIP(L)'s 'active site' prevents the intra-dimer cleavage of the heterodimer, which is therefore retained in the complex. (C) When the majority of adjacent dimers are heterodimers rather than homodimers, the FLIP(L)/caspase-8 heterodimeric enzyme will still be formed and process adjacent heterodimers as well as cleave co-recruited RIPK1; cleavage of RIPK1 prevents formation of the necrosome and therefore inhibits RIPK1-mediated necroptosis.

via necroptosis, FLIP (*Cflar*) knockout mice died as a consequence of caspase-8 activation and subsequent apoptosis [50]. Conditional knockout systems in mice have also aided the characterization of FLIP function in adult tissues. For example, in one study, the FLIP/Caspase-8 complex was shown to have a pro-survival effect and maintain homeostasis in the gut. Deletion of *Cflar* or *Casp8* led to enterocyte death and subsequent TNFα-dependent inflammation. In contrast to *Casp8* deletion, co-deletion of the gene encoding RIPK3 did not rescue this phenotype in conditional *Cflar* knockouts. These results and others demonstrate that the caspase-8/FLIP heterodimer is required to prevent necroptosis in the gut and that the presence of FLIP is also required to prevent caspase-8-dependent apoptosis [51–53]. In another study, tissue-specific deletion of *Cflar* in the epidermis of mice led to a severe inflammation. In these mice, loss of FLIP resulted in the production of autocrine TNFα and apoptotic cell death. Importantly, examination of the epidermis of patients with toxic epidermal neolysis (TEN) (characterized by erosive lesions of the skin, conjunctiva and mucous membranes as a result of epithelial cell apoptosis) expressed significantly reduced levels of FLIP when compared with healthy epidermis, thus resembling the phenotype observed in mice. TEN results

from severe reactions to drugs, in particular allopurinol, which is used clinically as a prophylactic treatment for gout and also hyperuricaemia associated with chemotherapy [54,55]. Therefore, therapeutic strategies modulating FLIP activity will need to consider these potential adverse interactions with commonly used drugs.

Murine models have also provided some clarity on the role of FLIP in the development of the immune system. For example, thymic-specific deletion of *Cflar* resulted in loss of single positive ($CD4^+$ or $CD8^+$) thymocyte subsets, suggesting that FLIP is required for the maturation of T cells from the double-positive stage of development to the single-positive stage [56]. In addition, several groups have reported the requirement for FLIP for the survival of mature T cells [57–59]. Therefore, in the thymus at least, FLIP seems to promote the development of a competent adaptive immune system. The role of FLIP in the tumour immune microenvironment is discussed in detail in section 5.3 of this review.

Noncanonical FLIP biology

In addition to its well-established roles in regulating procaspase-8 activation at the DISC, Complex-II and ripoptosome, FLIP has also been reported to be involved in the regulation of a number of additional signalling pathways that regulate cell fate. Some of these are discussed below.

FLIP as a suppressor of autophagy

There are at least two molecular mechanisms by which FLIP can attenuate autophagy (Fig. 2C). By interacting with autophagy related 3 (Atg3), FLIP inhibits the binding between Atg3 and microtubule-associated protein 1A/1B-light chain 3 (LC3) and, therefore, inhibits LC3 processing and autophagosome formation [60]. In addition, by interacting with procaspase-10, FLIP(L) has been reported to form an enzymatic complex in Multiple Myeloma (MM) cells that can cleave the Bcl-2-interacting protein Bcl-2 associated transcription factor 1 (BCLAF), which is a potent inducer of autophagy. This cleavage prevents BCLAF from displacing Bcl-2 from an inhibitory complex with Beclin-1, thereby preventing Beclin-1-induced autophagy [61]. It is unclear whether this is a FADD-dependent complex, although the interaction between procaspase-10 and FLIP(L) was reported as DED-dependent. MM cells require basal levels of autophagy for their survival, and it is proposed that the procaspase-10/FLIP(L) enzymatic complex tempers the levels of autophagy to avoid cell death induction; therefore, therapeutically disrupting or inhibiting this complex may induce autophagic cell death in MM.

FLIP/caspase-8 as regulators of the inflammasome

Inflammasomes are multi-subunit complexes into which inflammatory caspases are recruited and activated. These caspases then cleave and activate interleukin-1β and interleukin-18. The best-characterized inflammasome is the NLRP3 inflammasome, aberrant activation of which has been linked with various auto-inflammatory, auto-immune and chronic inflammatory diseases [62]. Using $RIPK3^{-/-}$, $RIPK3^{-/-}/FADD^{-/-}$ and $RIPK3^{-/-}/Caspase\text{-}8^{-/-}$ mouse models, it has been established that FADD and procaspase-8 are required for full activation of the NLRP3 inflammasome [63]. Procaspase-8 was shown to co-localize with procaspase-1 in LPS-treated macrophages, and both FADD and caspase-8 were shown to interact with core components of the inflammasome [63]. Further, procaspase-8 can bind directly to the critical NLRP3 inflammasome adaptor Apoptosis-associated speck-like protein containing a CARD (ASC) [25,64,65]. FADD and caspase-8 also play a critical role in the transcriptional upregulation of key inflammasome genes after LPS priming. In either $RIPK3^{-/-}/FADD^{-/-}$ or $RIPK3^{-/-}/Caspase\text{-}8^{-/-}$ mouse models, induction of inflammasome components was significantly reduced in response to LPS treatment when compared with $RIPK3^{-/-}$ control [45]. Similar to procaspase-8 and FADD, FLIP(L), through its pseudo-caspase domain, was found to interact directly with NLRP3 and procaspase-1, but was unable to interact with ASC. Loss of FLIP(L) inhibited pyroptosis, whereas its overexpression significantly increased the secretion of IL-1β. FLIP(L) was also found to bind the double-stranded DNA interacting HIN domain of the absent in melanoma 2 (AIM2) inflammasome directly and is required for its full activation [66,67].

FLIP and Wnt signalling

A number of groups have also reported a role for FLIP in the Wnt signalling pathway. Naito *et al.* [68] were the first to show that overexpression of FLIP(L) decreased the ubiquitination of β-catenin, stabilizing the protein and leading to its translocation to the nucleus causing an increase in Wnt signalling. This stabilization of β-catenin was FADD-dependent but independent of procaspase-8 [68]. Similarly, enhancement of β-catenin was reported following overexpression of the viral FLIP protein E8 [69]. By mutating the

nuclear localization signal in FLIP(L), thereby forcing cytosolic localization of the protein, FLIP(L)'s ability to modulate Wnt signalling was diminished, suggesting that its nuclear localization is important for this function [70]. More recent studies have revealed that, once in the nucleus, FLIP(L) can interact via its DEDs with the transcriptional regulator TIP49. Overexpression of FLIP(L) enhanced TIP49 binding to the *ITF2* promoter, a target of the β-Catenin transcriptional complex, and silencing of either TIP49 or FLIP(L), attenuated ITF2 expression, suggesting a role for FLIP(L) in Wnt signalling transcriptional complexes [71]. More generally, FLIP(L) has been reported to impair the UPS by forming aggregates in the perinuclear region of the cell, reducing ubiquitination of short-lived proteins such as HIF-1α and β-catenin [72].

FLIP and NFκB and MAPK signalling

In addition to their role in apoptosis, death receptors such as TRAIL-R2 are also reported to function in pro-survival signalling, proliferation, invasion and metastasis [73–78]. In the context of the Fas DISC, p43-FLIP(L) has been reported to interact with TNF receptor-associated factor (TRAF) 1 and 2 and the kinases RIPK1 and Raf-1 to activate NFκB and extracellular signal-regulated kinase (ERK) signalling [79]. Conversely, studies have reported that FLIP inhibits NFκB activation on engagement of the Fas receptor [80]. In addition to NFκB signalling, FLIP(L) can also affect the MAPK pathway; for example, in the context of TNFα signalling, FLIP(L) was shown to interact with MAP kinase kinase (MKK) 7, thereby inhibiting JNK-dependent ROS accumulation by preventing MKK7 binding to MAP/ERK kinase kinase 1 (MEKK1), apoptosis-signal-regulating-kinase 1 (ASK1) and TGF-β activated kinase 1 (TAK1) [81].

In addition to the canonical DISC-dependent cleavage of FLIP(L), a cytosolic cleavage fragment, termed p22-FLIP (Fig. 1A), has been described. This cleavage fragment is formed when the DEDs of FLIP(L) and procaspase-8 interact independently of the DISC. The endogenous catalytic activity of procaspase-8 is sufficient to cleave FLIP(L) at D198, forming p33 and p22-FLIP cleavage products. As p22-FLIP still retains the tandem DEDs, it can then be recruited to active DISCs where it can inhibit apoptosis in a manner similar to FLIP(S)/(R). Interestingly, overexpression of this fragment was found to induce potent activation of NFκB [82]. Similarly to FLIP(L) and FLIP(S), TAK1 is required for p22-FLIP to activate NFκB. In contrast to FLIP(L), however, p22-FLIP and FLIP(S) do not require the linear ubiquitin chain assembly complex (LUBAC) for activation of NFκB, but rather appear to require FADD and RIPK1 [83]. In addition, unlike the vFLIP protein from Kaposi's sarcoma-associated herpesvirus (a potent NFκB activator), no direct interaction between cellular FLIP proteins (FLIP(L), FLIP (S) and p22-FLIP) and IKKγ/NEMO was found [83].

FLIP and the endoplasmic reticulum

Recently, FLIP(L) was shown to localize to the endoplasmic reticulum (ER) and mitochondria-associated membranes (MAMs), where it was found to attenuate caspase-8-mediated processing of reticulon 4 (RTN4). Subsequently, ablation of FLIP led to distinct morphological abnormalities in the ER, including attenuated Ca^{2+} release and a reduction in tethering of mitochondrial membranes to the ER [84]. *Cflar*$^{-/-}$ MEFs exposed to starvation conditions showed a marked increase in lipid biogenesis and droplet formation compared with wild-type controls, suggesting that FLIP may also play a role in lipid accumulation and adaptation to stress [85]. Surprisingly, however, a recent report showed loss of FLIP actually increased a cell's resistance to ER stress. In the absence of FLIP, the PERK and IRE-1 ER stress pathways were found to be compromised due to enhanced Akt activity. Restoring FLIP expression was sufficient to re-sensitize the cells to ER stress and so, in this context at least, FLIP appears to have a pro-death role [86]. Interestingly, ER stress was also shown to downregulate FLIP, sensitizing cells to PERK-dependent upregulation of TRAIL-R2 [87].

FLIP regulation

FLIP splice forms are regulated at the transcriptional, translational and post-translational levels by various stimuli in a cell-specific manner. The multiple levels at which splice form-specific expression of FLIP is controlled underline the fundamental importance of these proteins in regulating cell fate.

Transcriptional regulation

CFLAR gene expression is regulated by a host of different transcription factors. Prominent among these factors is NFκB [88], a critical downstream effector of several signalling cascades, including TNFR1 signalling, where NFκB-mediated upregulation of FLIP promotes a pro-survival response by inhibiting cell death mediated by TNFR1 Complex-II [38]. In addition, FLIP transcription has been reported to be upregulated by (among others) CREB [89], NFATc2

[59], EGR1[90], androgen receptor [91–93], SP1 [94] and p63 [95]. *CFLAR* gene expression is also repressed by a number of transcription factors, including c-Fos [96], c-Myc [97], FoxO3a [98], IRF5 [99], E2F1 [100] and SP3 [101]. The proto-oncogene c-Fos was shown to act in a pro-apoptotic manner in prostate cancer cells by lowering transcription of FLIP(L), thereby sensitizing these cells to TRAIL treatment [96]. Moreover, the transcription factor c-Myc, a target of multiple pathways including Wnt, MAPK signalling, TGFβ and T cell receptor pathways, was shown to directly repress FLIP mRNA expression and induce TRAIL sensitivity in both cell lines and murine models. Using luciferase reporter assays and ChIP analysis, it was shown that c-Myc directly binds to the FLIP promoter to effect this change [97]. A recent paper from the Letai lab proposed that highly proliferative tissues with high c-Myc activity (as is found in many cancers) are apoptotically primed; in this regard, c-Myc-mediated repression of FLIP expression may contribute to this apoptotic priming [102]. In conclusion, FLIP is regulated by multiple, interconnected pathways providing potential opportunities for indirect modulation of FLIP by agents targeting the relevant pathways in specific tissues/cancers (*see below*).

Post-transcriptional regulation

FLIP proteins are generally short-lived proteins, with the half-lives of FLIP(S) and FLIP(L) reported to be ~ 30 min and ~ 3 h, respectively. The turnover of both proteins is regulated by the ubiquitin-proteasome system (UPS). Although FLIP(S) lacks the caspase-like C-terminal domain of FLIP(L), both proteins share some C-terminal (non-DED) amino acid residues known to be modified post-translationally [103–105]. K192 and K195 were identified as key ubiquitin acceptors and regulators of FLIP(S) turnover via the UPS. Although FLIP(L) shares these residues, mutation of K192 and K195 in FLIP(L) did not affect the stability of this splice form [103]. Similarly, in a study identifying S193 on all FLIP splice forms as a target of PKCα/β-dependent phosphorylation, the effect on FLIP's stability was splice form-specific [105]: phosphorylation of S193 led to decreased ubiquitination of all FLIP isoforms; however, this only enhanced the stability of FLIP(S) and FLIP(R), whereas no effect on FLIP(L) stability was observed.

Using FLIP(L) overexpression constructs, T166 phosphorylation and K167 ubiquitination were identified as key mediators of ROS-dependent FLIP downregulation [104]. In addition, it has been reported by several groups that Fas receptor engagement leads to production of nitric oxide (NO), which inhibited Fas-mediated apoptosis [106–108]. The NO was shown to induce S-nitrosylation (a covalent linkage of NO to cysteine residues) of Cys 254 and Cys 259 of FLIP(L), resulting in the inhibition of its ubiquitination and increased stability [107,108].

TRAF2 is an E3-ubiquitin ligase and a known FLIP-interacting protein, and it has been reported that TRAF2 may be able to ubiquitinate FLIP. A recent paper described a multi-protein cytosolic complex termed the FADDosome formed following treatment of cancer cells with 5-Fluorouracil; this p53/ATR/Caspase 10- and TRAF2-dependent cell death mechanism relies on the ubiquitination of FLIP by TRAF2, leading to its degradation, caspase-8 activation and cell autonomous apoptosis [109]. Importantly, this was reported to be a cytosolic complex. However, previous studies have shown that TRAF2 specifically interacts with caspase-8-processed p43-FLIP(L) at the DISC, leading to its stabilization and subsequent pro-survival signalling [110]. Interestingly, other members of the TRAF family, TRAF6 and TRAF7, have also been reported to promote poly-ubiquitination of FLIP, suggesting a wider role for this family of E3 ligases in regulating the stability of FLIP [111].

Another E3 ligase, ITCH, has been reported to directly interact with and ubiquitinate FLIP(L) leading to its degradation and sensitization of cells to TNFα-induced cell death and DNA damage [112,113]. However, ubiquitination is a reversible process and a family of proteins collectively known as deubiquitinases (DUBs) can cleave ubiquitin from proteins preventing their degradation, re-localization or other ubiquitin-related signalling effects [114]. Until recently, a specific DUB targeting FLIP had not been identified; however, a screen of 32 DUBs identified USP8 as a major moderator of FLIP(L) ubiquitination. Further assessment revealed direct binding of USP8 between amino acids 203-408 of FLIP(L) and confirmed that the interaction occurs at endogenous expression levels. Further, USP8 overexpression was able to negatively regulate death receptor-mediated apoptosis, in line with the observation that it can stabilize FLIP(L) [115]. Of note, although USP8 was determined to interact specifically with FLIP(L), it was also reported to have a role in regulating the stability of FLIP(S). Paradoxically, overexpression of USP8 led to increased ubiquitination of FLIP(S), leading to its degradation via the UPS, suggesting that the stabilizing effect of USP8 was through an intermediary protein [116]. Subsequent work suggested that ITCH is the intermediary E3 ubiquitin ligase [116,117]. Thus, the USP8/ITCH axis may play a key role in determining the relative levels

of the two main FLIP splice forms. In addition, there is evidence that downregulation of FLIP via JNK activation of ITCH may be mediated by ER stress-dependent JNK/CHOP/TRAIL-R2 signalling (Fig. 2) [86,118,119].

FLIP expression and drug resistance in cancer

FLIP has been shown to be overexpressed in a number of cancer types, including non-small-cell lung cancer (NSCLC) [10], colorectal cancer [120–123], pancreatic cancer [124], nasopharyngeal carcinoma [125], stomach cancer [126,127], meningiomas [128], urothelial cancer [129], prostate cancer [93], acute myeloid leukaemia [130], Burkitt's lymphoma [131], cervical carcinomas [132,133], ovarian carcinoma [134] and breast cancer [135,136]. It is also well established that the viral FLIP K13 has antiapoptotic activity and plays an important role in the pathogenesis of gamma-herpesvirus HHV-8-associated tumours, including Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease [137–139]. Several themes have emerged that influence the impact of FLIP expression on cellular phenotype, including subcellular localization of the protein, splice form-specific effects and the stage of the tumour.

FLIP as a biomarker of prognosis

FLIP(L) has a bipartite nuclear localization signal (NLS) in its C-terminal region alongside a nuclear export signal (NES) (Fig. 1), allowing shuttling of the protein between the nucleus and the cytosol [70]. This, in effect, creates two distinct pools of FLIP. Immunohistochemistry is able to distinguish between these different pools and correlates not only the expression, but also the subcellular localization of FLIP protein with patient outcomes. In a cohort of 184 NSCLC patients, we recently found that high cytoplasmic but not nuclear FLIP significantly correlated with a ~ >2-fold decrease in overall survival [10]. In a separate study of 32 Burkitt's lymphoma cases, the authors identified a mainly cytoplasmic pool of FLIP that highly correlated with poor patient outcome [131]: 24% of patients showing cytoplasmic FLIP expression had an overall survival of over 2 years, whereas 93% of those without detectable cytoplasmic FLIP expression had an overall survival over 2 years.

Several studies have identified FLIP as an independent adverse prognostic indicator [123,126,129,134] and shown both FLIP(L) and FLIP(S) to be important in patient treatment outcomes [124]. However, there is evidence that the expression of specific splice forms of FLIP is critical in particular clinical contexts. In one study of 462 colorectal cancer patients, FLIP(L) was identified as an independent marker of poor prognosis, whereas FLIP(S) (and TRAIL receptor) expression did not show a significant correlation with prognosis [121]. In a study of 536 cervical carcinoma samples, FLIP(L) expression increased in line with the grade of the carcinoma, although no significant relationship between FLIP and patient survival could be established [133]. In addition, in a study by our group, FLIP(L) mRNA was shown to significantly correlate with poorer overall survival of a cohort of AML patients [130]. Thus, overall FLIP expression is frequently upregulated in multiple cancers and correlates with poor prognosis.

FLIP as a mediator of therapy resistance

One of the reasons behind the association between high FLIP expression and poor prognosis is that FLIP confers resistance to a number of therapeutics. In early studies in colorectal cancer, we demonstrated that high FLIP expression confers resistance to apoptosis induced by standard-of-care chemotherapeutic agents (5-Fluorourcail and oxaliplatin) *in vitro* and *in vivo* [140]. Moreover, siRNA-mediated downregulation of FLIP [particularly FLIP(L)] significantly enhanced chemotherapy-induced cell death. Similar results were found with cisplatin in NSCLC models, docetaxel in prostate cancer models and more recently in combination with ionizing radiation in NSCLC [9]. Similarly, increased levels of FLIP(S) induced by AKT/mTOR/S6K1 and the Ral effector protein (RalBP1) have been linked to inhibition of cisplatin and oxaliplatin-induced apoptosis [93,127,141–145].

In addition to mediating resistance to chemotherapeutics, high FLIP expression also confers resistance to apoptosis-targeted therapeutics. Not surprisingly, there is a wealth of data showing that FLIP is key determinant of resistance to TRAIL receptor agonists; however, it remains to be seen whether high FLIP expression will be a key clinical mechanism of resistance to the next generation of multi-valent TRAIL receptor agonists. We have also reported that FLIP is an important determinant of resistance to IAP antagonists, with FLIP preventing cell death induced in response to both monovalent and bivalent IAP antagonists by blocking caspase-8 activation at the ripoptosome [146,147]. More recently, we reported that acute induction of FLIP expression in response to MEK inhibition in *BRAF* mutant colorectal cancer results in resistance to MEK inhibitor-induced cell death [148]. Thus, FLIP can also confer resistance to molecularly targeted agents that do not directly engage the apoptosis machinery.

FLIP and the tumour immune microenvironment

Another potential reason for the correlation between FLIP expression and poor prognosis is that high tumoral FLIP expression can inhibit cell death induced by immune effector cells via the Fas and TRAIL death receptors. Indeed, FasL and TRAIL expressed by cytotoxic T lymphocytes and natural killer cells may exert immunological selection pressure for tumours with high FLIP expression. In support of this, overexpression of FLIP [149] or a viral homologue (KSHV-FLIP) [14] in tumours was sufficient to permit establishment of tumours in immune-competent mice by inhibiting Fas-dependent cell death initiated by CTLs on target tumour cells.

FLIP is also important in the immune compartment. Myeloid-derived suppressor cells (MDSCs) are immunosuppressive cells that are recruited to sites of nonresolving inflammation, including cancer [150], and correlate with poor prognosis in patients [133,151]. Two types of MDSCs, monocytic and polymorphonuclear, have been identified, and recently, FLIP was shown to be critical for the survival of monocytic MDSCs through its ability to inhibit extrinsic apoptosis [5]. In keeping with an important role for FLIP in maintaining local immunosuppressive environments, FLIP has recently been shown to be critically important in maintaining FoxP3$^+$ regulatory T cells (T_{regs}), while no difference was observed in the development of normal thymocyte subsets upon FLIP knockout [6]. Importantly, in dendritic cells, loss of FLIP was shown to induce higher MHC II expression on the cell surface, increased IL-2, GMCSF, TNFα expression and enhanced T-cell activation [4]. Therefore, in peripheral sites (i.e. outside the lymph nodes), FLIP seems to play an important role in the maintenance of an immunosuppressive milieu.

Therapeutic strategies to target FLIP

Indirect targeting

Current standard-of-care chemotherapies such as cisplatin, 5-Fluorouracil (5-FU), gemcitabine, etoposide and paclitaxel have been shown to downregulate FLIP expression in various tumour cell line models [124,141,143,152–155] (Table 1). In ovarian cancer cells, it was shown that following treatment with cisplatin, a ternary complex of p53/FLIP and ITCH forms, driving ubiquitination and degradation of FLIP. Interestingly, in this complex, ITCH was reported to be able to degrade both FLIP(L) and FLIP(S) [141,152]. In a subsequent study, it was shown that active Akt1 prevented the localization of p53/FLIP and ITCH into this complex and acted as a resistance mechanism to cisplatin-induced FLIP degradation [143]. Cisplatin has also been shown to decrease FLIP in other cancers including glioblastoma and pancreatic cancer [124,156] and oral squamous cell carcinoma [153]. Similarly, the antimetabolites 5-FU and gemcitabine have also been reported to downregulate FLIP [124,153] in colorectal and pancreatic cancer models, respectively [155]. The topoisomerase II inhibitor etoposide was also shown to downregulate FLIP and sensitize cells to CD95-mediated cell death [154]. Therefore, chemotherapeutics can downregulate FLIP; however, the mechanisms by which this occurs are varied and often ill-defined and/or cell type specific [140].

Many groups have reported a reduction in FLIP mRNA as a result of treatment with HDAC (histone deacetylase) inhibitors [157–159]; however, post-translational effects of HDAC inhibitors on FLIP expression have also been reported. In particular, a novel interaction between Ku70, a protein usually involved in nonhomologous end-joining DNA double-strand break repair, and FLIP was described by our group. Ku70 binds R122 of FLIP in DED2, a site shared by all splice variants of FLIP and a site not required for FLIP binding to procaspase-8 or FADD. The Ku70/FLIP interaction was dependent on Ku70 acetylation. On treatment with HDAC inhibitors, Ku70 was acetylated, causing its dissociation from FLIP, resulting in FLIP poly-ubiquitination and degradation via the UPS [160]. Recently, we indirectly demonstrated the therapeutic potential of targeting FLIP expression

Table 1. Some examples of agents reported to inhibit FLIP expression.

Agents	Mechanism of action	References
Cisplatin, gemcitabine	DNA damaging agents/Anti-metabolites	[124,141,152, 153, 156]
Etoposide	Topoisomerase II inhibitor	[154]
Vorinostat (SAHA), amurensin G	Histone deacetylase inhibitor	[147, 161]
Antisense oligonucleotides and siRNAs	Blockade of mRNA translation, and RNAi	[170]
5-fluorouracil (5-FU)	Thymidylate synthase (TS) inhibitor	[124,153]
LY294002, PIK75	PI3K inhibitors	[163,165]
SNS-032	CDK9 inhibitors	[163]
KN-93	CAMK II inhibitors	[167]

using Class-I HDAC (HDAC1/2/3)-selective inhibitors such as entinostat, which we found enhances sensitivity to TRAIL, cisplatin and ionizing radiation in a caspase-8- and FLIP-dependent manner in NSCLC models. In addition, entinostat overcame FLIP(L)-mediated resistance to MEK inhibitors in *BRAF* mutant colorectal cancer. Thus, the use of HDAC1-3-selective agents appears to be an effective way of targeting FLIP by downregulating its expression [148]. However, this is an indirect method of targeting FLIP that will affect the expression of a large number of genes; moreover, this strategy is not effective in every disease setting (D. B. Longley, unpublished observations). In addition, Class III HDAC inhibitors (i.e. Amurensin G) targeting SIRT1 were found to augment the cytotoxic and apoptotic effects of TRAIL on leukaemia cells by upregulating TRAIL-R2 and c-Myc and downregulating FLIP [161].

Another similar approach is the use of CDK9 inhibitors, which were shown to effectively downregulate FLIP and Mcl-1 expression in a panel of NSCLC cell lines; this resulted in hypersensitivity to TRAIL *in vitro* and *in vivo*, with impressive efficacy against orthotopic lung cancer xenografts [162,163]. Recently, inhibitors of the bromodomain and extra terminal domain (BET) family such as JQ1 have also been shown to effectively downregulate FLIP expression, again leading to enhanced sensitivity to TRAIL-induced cell death. In prostate cancer, the apoptotic effects of proteasome inhibitors was reported to be mediated by upregulation of TRAIL receptors and decreased FLIP expression as a result of AP-1-mediated transcriptional repression [164]. In addition, PI3K inhibitors have been found to downregulate FLIP protein and mRNA in prostate cancer cells, where androgens regulate FLIP in a PI3K/AKT-dependent manner [89,165,166]. In addition, Calcium/Calmodulin-dependent Protein Kinase II (CAMKII) was shown to modulate FLIP levels at the DISC by phosphorylating FLIP(L), leading to its efficient recruitment to the DISC and subsequent inhibition of Fas-induced apoptosis; therefore, CAMKII inhibitors may sensitize cancer cells to death receptor-mediated apoptosis [142,167,168]. A final potential approach would be to target the ubiquitin-specific proteases (USPs) responsible for inhibiting the turnover of FLIP via the UPS, such as USP8 as discussed above [115]. As a class, USPs are attracting considerable interest as anticancer drug targets.

A very interesting insight into FLIP biology that suggests a potential way of indirectly targeting it was provided by Olivier Micheau's group, who found that FLIP is a highly thermo-labile protein and that relatively mild heat shock is sufficient to cause FLIP aggregation and depletion from the cytosol [169]. This, in turn, inhibited FLIP recruitment to the DISC and thereby enhanced TRAIL-induced apoptosis. These results suggest that whole body or localized hyperthermia may be a novel way of overcoming FLIP-mediated inhibition of therapy-induced cell death.

Direct targeting

Beyond antisense approaches, which we previously explored the feasibility of [170], selectively targeting FLIP without also inhibiting its closely structurally related paralog caspase-8 is challenging. In 2014, we proposed a model of DISC assembly which diverged from the widely held view that FLIP directly competes with procaspase-8 for binding to FADD [171]. We showed that FLIP preferentially uses phenylalanine 114 at the α2/α5 interface of its 2nd DED to interact with histidine 9 in the α1/α4 of the DED of FADD. In contrast, procaspase-8 preferentially uses tyrosine 8 in the α1/α4 of its DED1 to interact with the α2/α5 of the DED of FADD at phenylalanine 25. This preferential binding orientation reveals a potential opportunity to selectively target FLIP, while maintaining the recruitment of procaspase-8 to the DISC and related complexes; a medicinal chemistry programme is underway to exploit this opportunity [172].

A similar approach was explored to develop peptides derived from FLIP's DED1 α2-helix and DED2 α4-helix that were able to specifically target the interaction between FLIP and Atg3 without affecting the Atg3-LC3 interaction. These peptides individually bound to FLIP or Atg3 and were shown to induce growth suppression and autophagic cell death [60]. These results suggest that peptide mimetics based on these regions of FLIP have potential as anticancer therapies.

Conclusions

When considering therapeutic targeting of FLIP, the potential effects of its inhibition on normal tissues should, of course, be considered. *Cflar* null mice die at E10.5 with endothelial defects due to unrestrained caspase-8-mediated apoptosis. FLIP expression in the intestine was also found to be required for development. Moreover, selective deletion of *Cflar* from the intestinal epithelium of adult mice caused severe tissue damage and inflammation, and the animals died within a few days. Mechanistically, this was again found to be due to activation of caspase-8. Furthermore, tissue-specific deletion of *Cflar* in skin results in severe inflammation and loss of barrier function due to

elevated levels of TNFα. FLIP has also been demonstrated to play important roles in B-cell and T-cell activation [51,54].

However, it should be remembered that complete genetic deletion is not the same as transitory pharmacological inhibition. The fact that agents that indirectly target FLIP such as entinostat (recently awarded breakthrough status by the FDA for aromatase inhibitor-resistant breast cancer) have a therapeutic window suggests that more specific FLIP-targeted agents will also be tolerated. Moreover, enhanced dependence on FLIP in cancer cells may be driven by the frequently observed high levels of procaspase-8 expression as well as overexpression of FADD, which is frequently co-amplified with cyclin-D1 on chromosome 11q13. In addition to *FADD* amplification, other mutations may promote FLIP 'addiction'. For example, Kim *et al.* found that NSCLC cells with *NLRP3* mutations are hypersensitive to loss of FLIP expression. This potentially identifies a biomarker-definable subgroup of patients who would be hyper-responsive to FLIP-targeted therapies. In this regard, somatic gain-of-function mutations in *NLRP3* are present in 16% of lung adenocarcinomas, 18% of melanomas, 15% of invasive breast carcinomas and 14% of serous ovarian cystadenocarcinomas (TCGA) [173].

Thus, there is evidence that a therapeutic window for directly targeting FLIP exists. Given its importance in both regulating survival of tumour cells and tumour-supporting immune cells, such agents are likely to have widespread therapeutic utility.

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